TRAP Induces More Intense Tyrosine Phosphorylation than Thrombin with Differential Ultrastructural Features

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We have analyzed modifications on platelet ultrastructural morphology, cytoskeletal assembly, and tyrosine phosphorylation developing in platelets activated by both thrombin and the thrombin receptor-activating peptide (TRAP). Washed platelets exposed to various concentrations of thrombin or TRAP, for different periods, were: fixed and examined by electron microscopy, or lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under similar activating conditions, thrombin and TRAP induced different sequences of activation causing distinctive morphological and biochemical changes. Platelets exposed to thrombin showed centralized organelles encircled by constricted microtubule coils and granules secreting their contents through narrow channels of the open canalicular system. In contrast, activation by TRAP induced swelling of the open canalicular system with organelles remaining randomly dispersed and microtubules peripherally distributed. Compared to thrombin activation, TRAP induced higher rates of actin polymerization; increased association of actin-binding protein, myosin, and α -actinin; and higher association of tyrosine-phosphorylated proteins with the insoluble cytoskeletal fraction. Secretion of intragranule substances, measured as expression of P-selectin and lysosomal integral membrane protein at the surface level, were similar for both agonists at equivalent concentrations. Our biochemical observations indicate that TRAP causes more intense changes in signaling through tyrosine phosphorylation of proteins associated with the cytoskeletal fraction than thrombin. However, as derived from ultrastructural observations, TRAP seems to be less efficient in triggering cytoskeletal assembly and internal contraction in an organized manner in contrast with the natural protease. (*Am J Pathol* 2002, 160:2245–2252)

Platelet response depends on the nature and degree of the activating stimulus. Thrombin is one of the most powerful platelet-activating agents and is able to promote a full range of platelet responses, including the increase in the concentration of cytoplasmic Ca^{++} , leading to the activation of signaling cascades with shape change, internal contraction, and secretion. Platelet response to thrombin activation is guaranteed by the presence of at least two types of receptors, with high and moderate affinity for thrombin. The former is located in the N-terminal domain of the GPIIb α ,^{1–3} and the later with moderate affinity belong to the G protein-coupled seven transmembrane domain superfamily, known as protease-activated receptors (PARs).^{4,5}

Cleavage of PAR-1 by α -thrombin yields an agonistic new amino terminus with the initial sequence SFLLRN. This peptide sequence, termed thrombin receptor-activating peptide (TRAP), can also activate this receptor. TRAP is able to induce aggregation and secretion by similar mechanisms than thrombin,⁴ it is also involved in inositol phosphatase metabolism,⁶ in inhibition of adenylyl cyclase,⁷ and in protein phosphorylation.⁸ Although it is generally accepted that TRAP acting on PAR-1 has the ability to induce many of the cellular responses observed after thrombin activation, comparative studies show that TRAP seems to be less efficient to induce the whole sequence of responses caused by thrombin.^{4,9–13} Considering that thrombin has an additional receptor on GPIIb α , differential responses to both agonists should be expected.

The present study has comparatively investigated morphological and biochemical features developing in platelets exposed to thrombin or TRAP. With this purpose in mind, we performed morphological studies at the ultrastructural level. Electrophoretic evaluation of the contractile proteins incorporated into the cytoskeletal fraction and of phosphotyrosine-related signaling mechanisms

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was also performed using whole platelet lysates and the polymerized cytoskeletal fractions.

Materials and Methods

Experimental Design

We studied the effects of thrombin and TRAP on platelet morphology, cytoskeletal assembly, and tyrosine phosphorylation of proteins. With this purpose, washed platelets were exposed to different concentrations of thrombin (range, 0.01 to 0.2 U/ml) or TRAP (range, 30 to 100 μ mol/L) for different periods (from 10 seconds to 180 seconds). After activation, platelet suspensions were: 1) fixed and examined by electron microscopy, 2) analyzed by flow cytometry to measure platelet secretion, and 3) lysed to resolve the protein content by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Western blotting.

Blood Sampling and Platelet Washing

Blood was obtained from healthy donors and collected into citrate/phosphate/dextrose at a final concentration of citrate of 19 mmol/L. Platelets were separated as platelet-rich plasma (120 g, 20 seconds) and washed three times with equal volumes of citrate/citric acid/dextrose (93 mmol/L sodium citrate, 7 mmol/L citric acid, and 140 mmol/L dextrose), pH 6.5, containing 5 mmol/L of adenosine and 3 mmol/L of theophylline.¹⁴ The final pellet was resuspended in a Hanks' balanced salt solution (136.8 mmol/L NaCl, 5.3 mmol/L KCl, 0.6 mmol/L Na_2HPO_4 , 0.4 mmol/L KH_2PO_4 , 0.2 mmol/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and incubated for 20 minutes at 37°C.

Aggregation Studies

Aggregation studies¹⁵ were performed using turbidimetric techniques (Aggrecoorder PA 3210 aggregometer Menarini Diagnostic, Firenze) in aliquots of platelet suspensions to determine equivalent doses of thrombin and TRAP. Concentrations ranged from 0.01 to 0.2 U/ml for thrombin and from 30 to 100 μ mol/L for TRAP.

Platelet Morphology in the Electron Microscope

Samples were prepared for evaluation at the electron microscope according to methods reported in detail in previous publications.^{16,17} Aliquots of washed platelets were activated with different concentrations of thrombin (0.01 to 0.2 U/ml) and TRAP (30 to 100 μ mol/L) and fixed at different times (10 seconds to 180 seconds). In some experiments, platelets were activated by TRAP (30 μ mol/L) followed by thrombin (0.1 U/ml) activation, and *vice versa*. Samples were combined with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3. After 15 minutes at 37°C, the samples were sedimented to pellets and the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. The cells were

then washed in buffer and combined with 1% osmic acid in veronal acetate. After exposure to the second fixative for 1 hour, the cells were dehydrated in graded series of alcohol and embedded in Epon 812.

Flow Cytometry Studies

Platelet secretion after activation of platelets with thrombin and TRAP was measured by flow cytometry in platelet suspensions before and after being incubated with 50 μ mol/L of cytochalasin B, and inhibitor of actin filament formation. Immunolabeling of platelets with the antibodies was performed using dual-color analysis, designed to minimize artifactual activation of the sample. Aliquots of 1×10^6 resting or activated platelets were added to polypropylene tubes filled with 50 μ l of phosphate-buffered saline (PBS), pH 7.2. Platelets were detected by using an anti-CD41 monoclonal antibody (clone FA6-152) conjugated with phycoerythrin (Pharmingen, San Diego, CA) at saturating concentrations and incubated in the dark, without stirring, for 15 minutes at room temperature. P-selectin and the lysosomal integral membrane protein (LIMP) were measured with anti-CD62P-FITC (clone CLBThromb/6) and anti-CD63-FITC (clone CLBGran/12) antibodies, respectively. An IgG₁-FITC monoclonal antibody (clone 679.1Mc7) was used as a negative control in all studies. Samples were diluted with 1 ml of PBS and analyzed immediately with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) at an excitation wavelength of 488 nm. Fluorescence and scatter signals were calibrated with 2- μ m Calibrite beads (Becton-Dickinson).

Platelets were differentiated by their characteristic forward *versus* side scatter and acquired by their positivity for anti-CD41. Histograms were composed from fluorescence data obtained in the logarithmic mode from 5000 events analyzed in each sample, using the CellQuest conversion software (Becton Dickinson) on a Macintosh Power computer (Apple Computer Inc.).

Data were expressed as the percentage of fluorescence-positive platelets. An analytical marker was set in the corresponding fluorescence channel to define 2% of the resting platelet population with the highest membrane fluorescence at the baseline level. This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above this level in all subsequent samples.

Preparation of Platelet Lysates and Cytoskeletal Proteins

Platelet suspensions were adjusted to 1.2×10^6 platelets/ μ l and divided into three aliquots, one was kept undisturbed at 37°C and the remaining were subjected to activation with 0.1 U/ml of thrombin and 30 μ mol/L of TRAP. To obtain platelet lysates, after 90 seconds of activation, aliquots were lysed by the addition of Laemmli's sample buffer (125 mmol/L Tris-HCl, 2% SDS, 5% glycerol, and 0.003% bromophenol blue), containing 2 mmol/L of sodium orthovanadate and 0.0625% *N*-ethylmaleimide, and heated at 95°C

for 5 minutes. Samples were kept frozen until electrophoretic analysis was performed.

Equivalent aliquots from nonactivated and activated platelet suspensions were processed to extract the platelet cytoskeletons, according to the procedure described by Jennings and colleagues¹⁸ with minor modifications.¹⁹ Nonactivated and activated samples were treated with an equal volume of lysis buffer (final pH 7.4) containing 2% Triton X-100, 100 mmol/L Tris-HCl, 10 mmol/L ethylene glycol bis (β -aminoethylether)-*N,N,N',N'*-tetraacetic acid, 4 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin, and 2 mmol/L sodium orthovanadate. The Triton-insoluble residues, corresponding to the polymerized cytoskeletal fraction, were isolated by sedimentation at $12,000 \times g$ for 5 minutes at 4°C in a microfuge. Samples were frozen at -40°C until electrophoretic evaluation was performed.

Analysis of Cytoskeletal Assembly

To evaluate the contractile proteins associated with the cytoskeleton, the protein content in samples corresponding to the cytoskeletal fraction was resolved on 7 to 12% SDS-polyacrylamide gels followed by staining with Coomassie brilliant blue R250. Stained protein bands were densitometrically analyzed as previously described,²⁰ using digital-video technology provided by a computerized image analyzer running specific software (Kodak Dytal Science 1D; Eastman-Kodak, Rochester, NY). Values of protein peak areas in the lanes containing Triton-insoluble residues from nonactivated platelets were considered as 100%. The association of certain protein with the thrombin-activated cytoskeleton was expressed as the percentage of increase over the amount of the same protein found in the respective lane corresponding to nonactivated platelets.

Analysis of Tyrosine-Phosphorylated Proteins

Proteins in platelet lysates and cytoskeletal fractions were resolved on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA).²¹ After blocking nonspecific binding, Western blots were probed with a horseradish peroxidase-conjugated anti-phosphotyrosine recombinant antibody RC20 (Transduction Laboratories, Lexington, KY). The excess of antibody was removed by extensive washing and blots were developed by the enhanced chemiluminescence method²² (Amersham Pharmacia Biotech, Essex, UK). Protein bands were densitometrically analyzed as mentioned before, and data expressed as the mean intensity of phosphorylation.

Statistics

Data are expressed as mean $\pm$ SE (SEM). Student's *t*-test data were used for statistical comparisons between data obtained after activation with thrombin and after activation with TRAP. A *P* level < 0.05 and *P* level < 0.01 were considered statistically significant.

Results

Ultrastructural Studies

Figure 1A shows the ultrastructural aspect of a washed resting platelet. The discoid form of resting platelets is supported by a circumferential coil of microtubules lying just below the surface membrane whereas α -granules and dense bodies are irregularly dispersed in the cytoplasm. After activation with thrombin, platelets undergo a full series of morphological changes.²³ Basically, early activated platelets lose their discoid form, become irregular, extend pseudopods, and undergo internal transformation. In more advanced stages of activation by thrombin, organelles become centralized, often appear degranulated, and encompassed by microtubule coils and apparently contracted bundles of actomyosin. Elements of the open canalicular system (OCS) are more prominent and occasionally filled with granule contents. Figure 1B summarizes the more prominent features in a platelet that has reached an advanced stage of activation.

Activation by TRAP caused some of the previously described findings although they did not seem to have a strict correspondence with those previously described in thrombin-activated platelets. Actually, activation by TRAP induced less extensive physical changes on platelets than those observed after thrombin. Early activation by TRAP caused formation of few pseudopodia, but platelet bodies remained basically spherical. The most remarkable finding is the swelling of the OCS. At further activation stages the swelling of the OCS persisted and progressed, organelles remained randomly dispersed, even though some were degranulated, and microtubule coils remained peripherally oriented. At late stages of activation, α -granules were degranulated and appeared swollen, making it difficult to determine whether they were residual α -granules or dilated portions of the OCS. Figure 1C illustrates some of the previously described features. Intriguingly, microtubule coils remained peripherally located throughout the process of activation. Figure 2 shows micrographs corresponding to early (Figure 2A) and late (Figure 2B) activation of platelets with TRAP. Images emphasize the immobility of the microtubule ring, the swelling of the OCS after activation with TRAP and how the system of membranes may become transformed into large vacuoles. Activation of platelets with TRAP followed by thrombin led to morphological features resembling those observed with thrombin alone (Figure 2C). Under these activating conditions, we did not observe swelling of the OCS, characteristic of activation with TRAP alone, and the microtubule coils and actomyosin filaments appeared organized toward the platelet center. Addition of TRAP to thrombin-activated platelets did not modify the ultrastructural changes induced by thrombin alone (data not shown).

Table 1 summarizes the differential ultrastructural information described before, but in a more intelligible format.

Evaluation by Flow Cytometry

The expression of intragranule proteins at the membrane level was measured after activation of platelets with 0.1 U/ml of thrombin or with 30 μ M/L of TRAP. After activa-

Figure 1. Electron micrographs showing morphological features of platelets before and after activation. **A:** Cross-section of a resting platelet. A circumferential ring of microtubules supports the discoid form. α -Granules (G) and dense bodies (DB) are irregularly distributed in the cytoplasm. Channels of the OCS connect with the surface membrane. **B:** Platelet exposed to 0.1 U/ml of thrombin for 1 minute. Platelets lose the discoid form, become irregular, and develop internal transformation. Granules are concentrated in the center and enclosed within a constricted ring of microtubules. **C:** Platelets exposed to 30 μ mol/L of TRAP for 1 minute. Activation by TRAP induces distension of the internal membrane system, with organelles randomly dispersed, although partially degranulated, and microtubule coils remain peripherally oriented. Original magnifications, $\times 36,000$.

Figure 2. Electron micrographs showing morphological features of platelets during early and late activation with TRAP. **A:** Platelets exposed to 30 μ mol/L of TRAP for 10 seconds. Platelets are slightly irregular, but organelles are dispersed and microtubule coils remain close to the surface. **B:** Platelets exposed to a higher concentration of TRAP (100 μ mol/L) for 60 seconds. Organelles are randomly dispersed and the microtubule coil remains close to the surface. Dilatation of the OCS and fusion with α -granules have produced a large vacuole. **C:** Platelets exposed to 100 μ mol/L of TRAP for 60 seconds followed by 0.2 U/ml thrombin for 1 minute led to morphological features resembling those observed with thrombin alone. Original magnifications, $\times 36,000$.

Table 1. Ultrastructural Features Taking Place after Platelet Activation with Thrombin and TRAP

	Thrombin	TRAP
Shape	Irregular with pseudopods	Predominantly spherical
Internal contraction	MT concentrated in platelet center	MT peripherally located
Degranulation	α -Granules fused with OCS, releasing their content	α -Granules/OCS dilated and fused together to cause release

MT, Microtubules; OCS, open canalicular system.

tion with thrombin, percentages of positive platelets for P-selectin and LIMP were $55.6 \pm 2.1\%$ (mean $\pm$ SEM, $n = 4$) and $20.9 \pm 1.8\%$, respectively. Activation with TRAP led to $46.1 \pm 2\%$ and $16.7 \pm 1.9\%$ of positive platelets for P-selectin and LIMP, respectively.

Similar experiments were performed in the presence of $50 \mu\text{mol/L}$ of cytochalasin-B. Under these conditions, both agonists were able to induce secretion measured as increases in the percentage of positive platelets for P-selectin and LIMP. In the presence of cytochalasin-B, values of percent of positive platelets were of $52.9 \pm 0.9\%$ (mean $\pm$ SEM, $n = 4$) and $20.8 \pm 1.2\%$ for P-selectin and LIMP, respectively, after thrombin activation, and of $44.5 \pm 1.1\%$ and $16.0 \pm 0.5\%$ for P-selectin and LIMP, respectively, after TRAP activation.

Modifications in the Incorporation of Cytoskeletal Proteins after Activation

Platelet activation with thrombin resulted in an augmented assembly of contractile proteins when compared to resting platelets (Figure 3A, lane 2 *versus* lane 1). The densitometric evaluation revealed increases of $135 \pm 8\%$ (mean $\pm$ SEM, $n = 4$), $70 \pm 5\%$, $35 \pm 4\%$, and $60 \pm 3\%$, for actin-binding protein (ABP), myosin, α -actinin, and actin, respectively, to the cytoskeletal fraction after thrombin activation (Figure 3B).

Activation of platelets with TRAP, used at concentrations equivalent to those causing a similar aggregation response to that induced with thrombin ($30 \mu\text{mol/L}$ of TRAP *versus* 0.1 U/ml of thrombin), caused a more marked incorporation of cytoskeletal proteins when compared to thrombin activation (Figure 3A, lane 3 *versus* lane 2). The densitometric evaluation detected increments up to $170 \pm 7\%$ (mean $\pm$ SEM, $n = 4$), $93 \pm 3\%$, $67 \pm 6\%$, and $103 \pm 10\%$, for ABP, myosin, α -actinin, and actin, respectively, with respect to values in resting platelets. Differences among results reached statistical significance when comparing TRAP-activated platelets *versus* thrombin-activated platelets ($P < 0.05$ for ABP and $P < 0.01$ for the rest of proteins).

Changes in Tyrosine Phosphorylation of Proteins

Studies on Whole Platelet Lysates

Both thrombin and TRAP triggered phosphorylation of proteins at tyrosine residues. Proteins p120, p100, p90, p62, p60, and p58 were clearly phosphorylated after

activation with both agonists (Figure 4A, lanes 2 and 3 *versus* lane 1). The densitometric analysis of Western blot profiles corresponding to whole platelet lysates did not show major differences among intensities of phosphorylation achieved with thrombin or TRAP used at equivalent concentrations. As represented in bar diagrams depicted in Figure 4B, activation of platelets with thrombin increased the intensity of phosphorylation from 73.9 ± 9 arbitrary units to 99.1 ± 16 arbitrary units after thrombin, and to 93.2 ± 24 arbitrary units after TRAP activation (mean $\pm$ SEM, $n = 4$).

Studies on Triton X-100 Insoluble Cytoskeletal Fractions

When analyzing the cytoskeletal fractions, we observed that activation with either thrombin (0.1 U/ml) or TRAP ($30 \mu\text{mol/L}$) induced phosphorylation of proteins p120, p100, p62, p60, and p58, proteins that seemed associated with the polymerized cytoskeleton. In contrast with the previous findings in whole platelet lysates, the intensity of phosphorylation of these proteins was greater after activation with TRAP (Figure 5A, lane 3 *versus* lane 2). The densitometric evaluation showed that exposure of platelets to equivalent concentrations of thrombin and TRAP increased the intensity of phosphorylation from 61.1 ± 15.6 arbitrary units to 92.3 ± 5 arbitrary units and 120 ± 10 arbitrary units, respectively. Differences among results reached statistical significance ($P < 0.05$ for TRAP *versus* thrombin, mean $\pm$ SEM, $n = 4$) (Figure 5B).

Discussion

The mechanisms of platelet activation by thrombin remain controversial, particularly because of the presence of several receptors, GPIb and PARs, with different affinities. The use of TRAP has allowed the study of the effect of activating the moderate affinity receptor alone. The present study was designed to analyze ultrastructural and biochemical changes produced by both thrombin and TRAP on platelet activation. Our findings demonstrate that activation with TRAP produces a similar biochemical response on platelets, but fails to induce the full sequence of morphological transformations observed with thrombin.

Platelet activation by thrombin starts a cascade of signaling events, which have a rapid translation into morphological changes,^{24,25} as confirmed in the present study. Interestingly, the ultrastructural findings observed in platelets activated by TRAP markedly differed from those observed when thrombin was used as the activating agent. The most remarkable differences consisted of

Figure 3. A: Coomassie blue-stained SDS-polyacrylamide gel electrophoresis profiles corresponding to the cytoskeletal proteins of resting platelets (C), platelets activated with 0.1 U/ml of thrombin (T), or with 30 μ mol/L of TRAP (TRAP) for 1 minute. **B:** Bar diagram representing increases in the association of the main contractile proteins: ABP, myosin, α -actinin, and actin. Results are expressed as a percentage of increase of each protein over the amount of the same protein found in profiles from nonactivated platelets. Profiles are representative of four different experiments (mean $\pm$ SEM; **, $P < 0.01$ and *, $P < 0.05$ for TRAP versus thrombin).

swelling of α -granules and dilation of the OCS. Moreover, formation of large vacuoles and diminished centralization of microtubule coils were consistently observed after TRAP activation. However, results from the present study confirm previously published work^{9,26,27} indicating that TRAP is as effective as thrombin in producing secretion in

Figure 4. A: Electrophoretic profiles corresponding to tyrosine-phosphorylated proteins in lysates from nonactivated platelets (C), and platelets activated with 0.1 U/ml of thrombin (T) or with 30 μ mol/L of TRAP for 1 minute. Some tyrosine-phosphorylated proteins are identified according to their molecular weights. **B:** Bar diagrams representing values of intensity of protein phosphorylation (mean $\pm$ SEM, arbitrary units, $n = 4$) in profiles from resting platelets (C) and platelets activated with 0.1 U/ml of thrombin (T) or with 30 μ mol/L of TRAP. Profiles are representative of four different experiments.

platelets. In addition, secretion induced by both effectors seems to be independent on cytoskeleton, because it was not modified by the presence of an inhibitor of actin filament formation such as cytochalasin.

Flow cytometry results and ultrastructural evaluation performed through the present study suggest that secretion could be accomplished through different mechanisms: actively, with exocytosis produced by membrane

Figure 5. **A:** Electrophoretic profiles showing tyrosine-phosphorylated proteins associated with the Triton-insoluble cytoskeletal fraction, from non-stimulated platelets (C) and platelets activated with 0.1 U/ml of thrombin (T) or 30 μmol/L of TRAP. **B:** Bar diagrams representing values of intensity of protein phosphorylation (mean ± SEM, arbitrary units, $n = 4$) in profiles from resting platelets (C) and platelets activated with 0.1 U/ml of thrombin (T) or with 30 μmol/L of TRAP. Results shown are representative of four different experiments (mean ± SEM; *, $P < 0.05$ for TRAP versus thrombin).

fusion and cytoskeletal contraction; and passively, with exocytosis produced by fusion of membranes. Although the first mechanism seems to be more efficient, the second one seems to be at least as effective in terms of P-selectin and LIMP expression. From electron microscopy, thrombin seems to induce the first mechanism, which occurs through organized cytoskeletal assembly.

On the contrary, ultrastructural analysis indicated that secretion in response to TRAP activation might occur without microtubule coil contraction. It is interesting to point out that the electrophoretic analysis of the cytoskeletal fraction revealed that TRAP was able to produce an apparent increase in the assembly of contractile proteins. However, this increased polymerization of cytoskeletal proteins did not seem to occur in an organized manner in response to TRAP activation because we did not observe centralization of the microtubule coil.

Our present results indicate that TRAP seems to be less efficient in causing the full sequence of morphological transformation. However, TRAP induced more intense biochemical responses than thrombin as assessed by a higher intensity of tyrosine phosphorylation of proteins and a more intense actin assembly. Several authors have suggested that TRAP is only a partial agonist because it induces less expression of activated GPIIb-IIIa, fails to induce platelet prothrombinase activity, and causes weaker responses in terms of Ca^{++} mobilization, arachidonate production, and serotonin release.^{8,9,28,29} Our studies do not fully support these concepts. In our hands, concentrations of thrombin and TRAP that induced similar aggregation responses in washed platelets resulted in nearly equivalent levels of phosphorylation at tyrosine residues of proteins present in whole platelet lysates. However, a marked increase in phosphotyrosine proteins was noted when analyzing the insoluble cytoskeletal fractions. These data imply that different signaling pathways could be involved in the activation induced by thrombin or TRAP, a concept that seems to be accepted in additional literature.^{2,30}

It may be argued that the differences noted in our activation studies could be related to the difficulty in achieving equivalent concentrations with the two activating agents. It is worth mentioning that concentrations of thrombin or TRAP that induced similar aggregating responses (ie, 0.1 U/ml of thrombin and 30 μmol/L of TRAP) caused equivalent levels of secretion and of phosphorylation of those proteins present in whole platelet lysates, but still induced differential intensity of phosphorylation of proteins associated with the insoluble cytoskeletal fractions.

Our studies on the assembly of proteins to the low-speed cytoskeletal fraction of activated platelets confirm that TRAP is more effective than thrombin in causing association of structural proteins into the insoluble pellets. In fact, this quantitative change in the presence of cytoskeletal proteins could partially explain the increased phosphorylation noted in our studies. It is commonly accepted that the platelet cytoskeleton plays an important role in platelet shape change, internal transformation, and secretion.^{18,31-33} The coordinated regulation of actin polymerization acting together with a balanced organization and incorporation of other cytoskeletal proteins are the key events for external and internal motile responses of platelets.^{34,35} Our present observations with thrombin and TRAP suggest that the latter agent induces a more intense association of polymerized actin and other cytoskeletal proteins, but does not result in an efficient internal contraction. This impression is supported by the consistent ultrastructural observation of microtubules more peripherally located in platelets activated

by TRAP. This discrepancy would further support the concept of different activating pathways being switched on by the two agonists.

It has been repeatedly suggested that both GPIb complex and PARs are required to ensure the maximal rate and extent of thrombin-induced platelet activation, as measured by increases in intraplatelet Ca^{++} concentrations. Both receptors seem to act simultaneously although independently.^{2,30} However, the ultrastructural features observed with TRAP do not seem to be physiological. Our present study further supports these concepts. In fact, studies performed in the present work demonstrated that addition of thrombin to TRAP-activated platelets caused completion of internal transformation identical to thrombin alone. Moreover, addition of TRAP to thrombin-activated platelets had no effect on morphology. These results suggest that activation of the receptor associated with GPIb is essential for the full effect of thrombin on platelets, and this is not achieved by TRAP alone.

In conclusion, our work suggests that thrombin and TRAP induce different sequences of activation causing distinct morphological and biochemical changes. Although TRAP seemed to induce a more intense association of the cytoskeletal proteins, and secretion of activation-dependent antigens, it did not produce internal contraction in an organized manner, failing to promote the full sequence of morphological transformations observed with thrombin.

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